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Whole-Somite Rotation Generates Muscle Progenitor Cell Compartments in the Developing Zebrafish Embryo

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SUMMARY

Somites are transient, mesodermally derived structures that give rise to a number of different cell types within the vertebrate embryo. To achieve this, somitic cells are partitioned into lineage-restricted domains, whose fates are determined by signals secreted from adjacent tissues. While the molecular nature of many of the inductive signals that trigger formation of different cell fates within the nascent somite has been identified, less is known about the processes that coordinate the formation of the subsomitic compartments from which these cells arise. Utilizing a combination of vital dye-staining and lineage-tracking techniques, we describe a previously uncharacterized, lineage-restricted compartment of the zebrafish somite that generates muscle progenitor cells for the growth of appendicular, hypaxial, and axial muscles during development. We also show that formation of this compartment occurs via whole-somite rotation, a process that requires the action of the Sdf family of secreted cytokines.

INTRODUCTION

Within vertebrate embryos, segmentation of the paraxial mesoderm during somitogenesis results in the formation of distinct anterior/posterior cellular compartments within the early somite, defined by differential gene expression. A secondary series of cellular rearrangements generates morphologically distinct, lineage-restricted somitic compartments that are, in turn, induced to form the progenitors of distinct tissues during embryogenesis. Fate-mapping studies, chiefly in the chick embryo, indicate that the dorsal aspect of the amniote somite produces an epithelial intermediary structure, the dermomyotome, that gives rise to the progenitors for skeletal muscle of the axis (the myotome) and to progenitors at limb levels, which are precursors of the appendicular muscles. The dermomyotome is also the source of resident adult skeletal muscle stem cells, the satellite cells (Christ and Ordahl, 1995; Gros et al., 2005; Relaix et al., 2005; Kassar-Duchossoy et al., 2005; Schienda et al., 2006). Dorsally, the dermomyotome generates the dermal layer of the forming skin of the back (reviewed in Brand-Saberi and Christ, 2000; Olivera-Martinez et al., 2000; Ben-Yair and Kalcheim, 2005). The ventral aspect of the amniote somite gives rise both to sclerotome, the progenitors of the axial skeleton, and the syndetome, which generates the tendons of the body axis (Brent et al., 2003; Christ and Ordahl, 1995).

By contrast, zebrafish somites show little overt morphological compartmentalization, and cell lineage analysis has defined the origins of only a subset of the cell types derived from the amniote somite. The best-described zebrafish somitic lineage is that of the embryonic myotome. Myoblast-specific gene expression is initiated coincidently with the anterior/posterior restriction of segmental gene expression with zebrafish somites (Weinberg et al., 1996; Coutelle et al., 2001). Two distinct myogenic compartments are discernable within presegmentation- and segmentation-stage zebrafish embryos and are generated by different processes. Adaxial cells are early differentiating progenitors of the slow twitch muscle lineage that arise from the most medial portion of the paraxial mesoderm and migrate to form a subcutaneous layer of slow twitch muscle at the lateral-most surface of the myotome (Devoto et al., 1996; Cortes et al., 2003). A second identifiable compartment is constituted by the cells of the lateral somitic mesoderm, which are induced to undergo myogenesis by a distinct set of signals from those that regulate adaxial cell myogenesis (Groves et al., 2005). Fate-mapping studies (Devoto et al., 1996) have revealed that lateral somitic cells contain progenitors of the fast twitch muscle lineage, although this previous analysis failed to provide evidence for any anterior/posterior restriction of the fast twitch muscle precursors within nascent somites, suggested by the restricted onset of myogenesis within the posterior domain of the lateral somite (Devoto et al., 1996; Weinberg et al., 1996). Sclerotomal cells are generated from ventral somitic regions, as in the amniote somite, but they represent a much-reduced proportion of the somitic derivatives (Morin-Kensicki and Eisen, 1997).

Despite these studies, the embryonic origins of other fish somitic derivatives remained undefined, leading to

the suggestion that the lineage-restricted compartments evident within the amniote somite may be innovations that arose during tetrapod evolution (Brand-Saberi and Christ, 2000; Stickney et al., 2000; Sporle, 2001; Hollway and Currie, 2003). In such a scenario, the increased reliance on the use of appendicular muscle evident in tetrapod, limb-dominant, locomotor strategies and the necessity for a defined embryonic dermal layer as an adaptation to terrestrial environs may have driven the evolution of a distinct dermomyotomal compartment. Conversely, other authors have argued, based only on the expression of specific genes, that an equivalent of the amniote dermomyotome does exist in fish species (Devoto et al., 2006; Steinbacher et al., 2006; Feng et al., 2006; Hammond et al., 2006). Equally unclear is the cellular basis for the prodigious growth evident within the larval zebrafish myotome, and it remains unknown if cells directly analogous to satellite cells exist within the zebrafish myotome. More generally, while the concept of recognizable, lineage-restricted compartments within the somites of different model systems is well defined, the molecular mechanisms by which these compartments arise from nascent somites is far from understood in any context.

In this study, we define a distinct lineage-restricted domain within the zebrafish somite, the anterior somitic compartment, which gives rise secondarily to a number of different cell types, including a series of muscle progenitor populations that are required during different phases of muscle growth. Generation of these somitic subdomains occurs through the mechanism of whole-somite rotation, a process that we show to require the activity of the secreted cytokine Sdfla and its receptors.

RESULTS

Molecular Definition of the Anterior Somitic Compartment

Analyses of the expression of the myogenic marker gene *myoD* within the zebrafish embryo reveals that it is initially restricted to the posterior region of newly formed somites, but by late somitogenesis encompasses the entire anterior/posterior extent of the somite, an expression profile shared by a number of other myogenic regulatory genes (Figure S1; see the Supplemental Data available with this article online) (Weinberg et al., 1996; Coutelle et al., 2001). This suggests that the anterior somitic domain does not contribute to the initial phase of myogenesis within the zebrafish somite. Furthermore, the expression of zebrafish orthologs of *pax3 pax7* and *dachshundD* (*dacD*), genes known to be expressed within amniote dermomyotome and hypaxial muscle progenitors (Gros et al., 2005; Relaix et al., 2005; Sporle, 2001; Bober et al., 1994; Heanue et al., 1999), is restricted to the cells of the anterior somitic compartment (Figure 1).

The anterior restriction of the expression of these genes is followed by a shift of expression domains. Both *pax3* and *pax7* come to be expressed in a single layer of cells superficial to the myotome at the end of somitogenesis, an expression profile that resembles the "external cell" population previously noted by other authors (Waterman, 1969; Groves et al., 2005; Devoto et al., 2006; Feng et al., 2006; Hammond et al., 2006) (Figures 1A–1H; Movie S1 [available in the Supplemental Data]). A distinct laterally progressing expression pattern for both of these genes precedes expression within the external cell layer, which suggests that *pax7*/*pax3*-expressing anterior somitic cells undergo an anterior-to-lateral rotation (Figures 1A, 1B, and 1L and Figures 5Q, 5T, and 5W). Furthermore, mitotically active Pax7-positive nuclei can be found at the external cell position, concentrating toward the horizontal myosepta, throughout larval, juvenile, and adult stages (Figures 1I–1K; Figure S2; data not shown). Pax7-positive nuclei can also be found localized between myofibers deep within the myotome from 3 days postfertilization (dpf) onward, underneath the basal lamina of individual fibers (Figures 1K; Figure S2). The majority of these cells are mitotically inactive, suggesting that fiber-associated Pax7 cells are quiescent (Figure S2). An identical niche and proliferative behavior is exhibited by mammalian satellite cells, which similarly express and also require Pax7 for their maintenance (Gros et al., 2005; Oustanina et al., 2004; Relaix et al., 2005; Seale et al., 2000).

The expression of *dacD* differs from that of the two *pax* gene orthologs, and this expression is restricted to the ventral/lateral edge of rostral somites (Figure 1U). This region has been shown to contain pectoral fin and hypaxial myoblast precursors, suggesting that the expression of *dacD* may specifically mark the progenitors of these cells (Neyt et al., 2000) (Figures 1Q–1U). In support of this notion, both *pax3* and a second *dac* ortholog, *dacA*, are expressed in migrating pectoral fin and hypaxial muscle precursors (Hammond et al., 2002) (Figures 1M–1P). To summarize, the zebrafish orthologs of amniote genes expressed within the dermomyotome, hypaxial and migratory limb muscle precursors and muscle-specific stem cells are initially expressed in the anterior domain of the zebrafish somite.

Whole-Somite Rotation Generates the Lateral "External Cell" Compartment

In order to investigate the morphogenetic movements that control formation of the external cell layer, we undertook whole-somite imaging by using the vital fluorophore BODIPY-Ceramide, followed by confocal laser scanning microscopy of the intact cells of the myotome over a time course that encompassed two periods of somite development (Figure 2). The cells of newly segmented somites undergo little, if any, rearrangement relative to their neighbors (Figures 2A–2F). However, by mid-somitogenesis, somitic cells have initiated a set of morphogenetic behaviors that ultimately result in the rotation of the entire somite 90° from its initial position. As a result of this cellular rearrangement, anterior somitic compartment cells come to lie in a lateral external cell position, a process confirmed by lineage tracing of individual anterior somitic compartment cells (Figures 2G–2N; Movie S2). Furthermore, individual labeled cells can rotate at different rates,

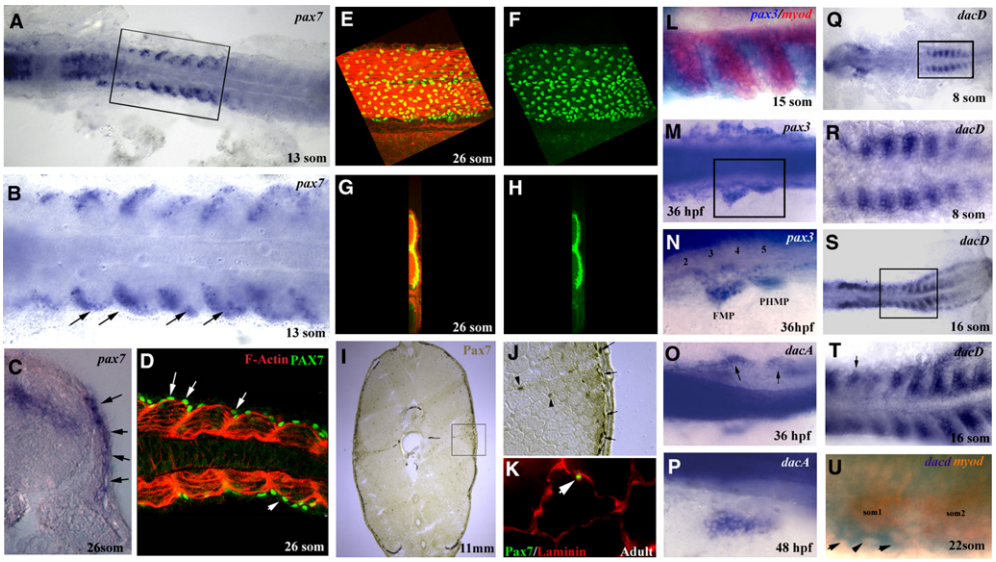

Figure 1. Genes Expressed in Hypaxial Muscle and Muscle Stem Cells Are Initially Restricted to the Anterior Somitic Compartment

(A) 13-somite embryo hybridized with an antisense probe to the *pax7* gene.

(B) High-magnification view of the region boxed in (A), revealing the restricted expression of *pax7* within anterior somites (arrows).

(C) Cross-section of a 26-somite embryo showing *pax7* expression restricted to a superficial layer of cells external to the myotome (arrows).

(D) A single confocal scan at the level of the notochord, in which filamentous actin within muscle cells (phalloidin, red) marks the extent of the myotome; Pax7-positive nuclei are marked in green (arrows).

(E–H) Maximum projection rendering of confocal Z-stacks through the entire mediolateral extent of a 4-somite length view of a 26-somite zebrafish embryo marked with Pax7 (green) and phalloidin (red) revealing that Pax7-positive cells constitute a single layer of cells superficial to the myotome (movie of the 3D projection provided as Movie S1). (G and H) Transverse projection views.

(I) Cross-section of an 11 mm juvenile zebrafish. Pax7-positive cells are found in a superficial "external cell" layer and adjacent to muscle fibers scattered throughout the myotome zebrafish (also see Figure S2).

(J) High-magnification view of the region boxed in (I). The highest concentration of Pax7 external cells (arrows) is found clustered at the horizontal myosepta; Pax7-positive cells are also clearly evident adjacent to individual muscle fibers deeper within the myotome (arrowheads).

(K) Cross-section of an adult zebrafish incubated with antibodies against Laminin (red), which marks the extent of the basal lamina of individual fibers, and Pax7 (green) reveals that Pax7-positive nuclei (arrow) reside underneath the basal lamina of individual fibers.

(L) Double in situ hybridization utilizing *pax3* (red) and *myoD* (blue) reveals that *pax3* expression becomes restricted to the anterior somitic compartment.

(M) *pax3* is also expressed in the migratory hypaxial muscle precursors of the pectoral fin muscles (FMP) and the posterior hypaxial muscle precursors (PHMP) (Haines et al., 2004).

(N) High-magnification view of the area boxed in (M). Numbers refer to somite position.

(O and P) *dacA* (Hammond et al., 2002) is highly expressed in (O) migratory and (P) postmigratory hypaxial muscles precursors.

(Q) *dacD* expression initiates in the anterior somitic compartment in newly segmented somites of an 8-somite embryo.

(R) High-magnification view of the region boxed in (Q), demonstrating the anterior somitic compartment restriction of this gene.

(S) Expression of *dacD* becomes restricted to lateral somite cells, as evident in rostral somites of this 16-somite-stage embryo.

(T) High-magnification view of the region boxed in (S), revealing the mediolateral progression (arrow), of expression of *dacD* at this stage of development.

(U) By the 22-somite stage, expression of *dacD* (blue) is confined to a region in the ventral layer, lateral to the differentiating myotome (arrows), here marked with *myoD* in red. Lateral view; anterior is oriented toward the left.

(A), (B), (D), and (L)–(T); dorsal views, anterior is oriented toward the left.

even when comparing neighboring cells that share an immediate lineage relationship (Figures 2M and 2N).

Fate of the Anterior Somitic Compartment

We next followed the fate of individual cells of the anterior somitic compartment, derived from different rostral/caudal levels within the embryo. Anterior somite cells derived from somite 4 migrate to contribute to pectoral fin muscle formation (Figures 3A–3F). Similarly, the anterior cells of somite 5 contribute to the posterior hypaxial muscle (PHM), which we previously showed is derived from the ventral regions of somites 5 and 6 (Haines et al., 2004) (Table S1). However, at these somitic levels, and at all other levels examined, a second fate was identified as originating from the anterior somite. Labeled cells migrate laterally to generate large, flat, Pax7-positive cells of the external cell layer (Figures 3G–3I and 4A–4F; Movies S3A and S3B; Table S1).

Fate-mapping strategies extended to later developmental periods also revealed that a subset of the external cell population generates muscle fibers 1 week after cells were initially labeled (Figures 3J–3M and 4G–4N; Table S1). Within lineage-related cell clusters, it is also possible to detect the original undifferentiated cell at the site of

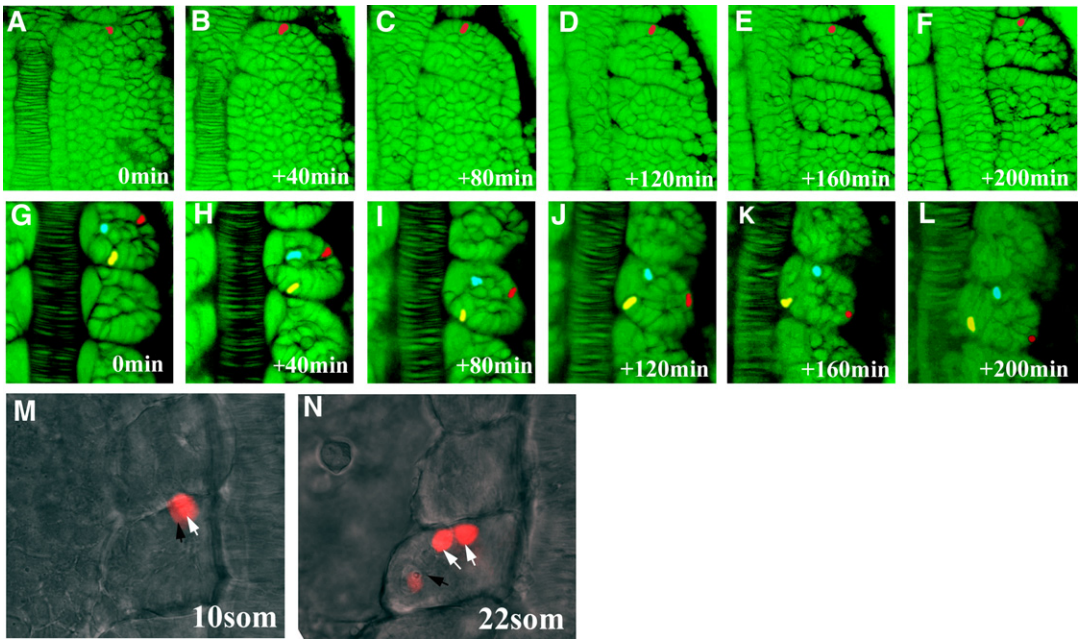

Figure 2. The Zebrafish Somite Undergoes Rotation

(A–F) Selected images of a single time-lapse confocal series of the early phase of somitogenesis in the zebrafish embryo, stained with BODIPY-Ceramide (green). Rostral somites are imaged during the formation of the first five somites, and a single cell (red) is imaged across this developmental period. Little or no movement of cells relative to their neighbors is evident.

(G–L) Selected images from a similar continuous time-lapse confocal series at the midphase of somitogenesis. Caudal yolk extension somites of a 15-somite-stage embryo are imaged, and individual cells (marked in red, blue, and yellow) are tracked over a 200 min period. Rotation of the right somitic field of cells is evident in a clockwise direction.

(M and N) Iontophoresis of the lineage tracer Rhodamine dextran (red) into anterior somitic compartment cells at the (M) 10-somite stage reveals that these cells rotate (counterclockwise in left-sided somites) to varying degrees during maturation of the somite ([N], 22-somite embryo). Black and white arrows track individual cells and their progeny.

All panels are dorsal views; anterior is oriented toward the top.

muscle fiber generation, an observation suggestive of stem cell self-renewal (Figures 3L and 4K–4N). A subset of the cells of the external cell layer generate larval muscle fibers and transit to fiber-associated positions deeper within the myotome, where they maintain Pax7 expression (Figures 4K–4N). The position of these Pax7-positive cells, medial to the superficial horizontal myosepta-associated external cell layer, suggests that these cells are satellite cells (Figures 1J, 1K, and 4K–4N; Figure S2). The remainder of labeled cells stay undifferentiated within the external cell layer at least 10 days after injection, the latest point at which labeled cells could still be identified. This is consistent with the observation that Pax7-positive external cells are evident within the postlarval myotome, suggesting that they represent a resident progenitor cell population required for muscle growth through the entire life of the fish (Figures 1I and 1J; Figure S2).

A third set of labeled anterior somitic cells migrated both dorsally and ventrally to a distinct subepidermal/epidermal-intercalated position consistent with a dermal layer (Figures 3R–3U; Table S1). Anterior somitic compartment cells also contributed to the formation of the dorsal fin when labels were made within somites inclusive and caudal to somite 6 (Figures 3N–3Q; Table S1).

Collectively, these results suggest that the anterior somitic cells of rostral somites generate seven distinct fates; external cells, appendicular and hypaxial muscle precursors, muscle progenitor cells that generate fibers during a secondary period of larval muscle growth, satellite cells, dermal cells of the skin, and cells contributing to the dorsal fin.

We also examined the fates of anterior somitic compartment cells derived from caudal somitic levels. Anterior somitic cells derived from somites 14–16 of 16-somite-stage embryos do not contribute to hypaxial muscle, although external cells, larval muscle fibers, and dermal and dorsal fin contributions are observed similarly to labels made within anterior somitic cells of rostral somites (Table S1). A distinct fate is derived from anterior cells of caudal somites, superficial embryonic fast twitch muscle fibers, evident 24 hr after labeling (Figures 3V–3Y). These results suggest that at caudal somitic levels, anterior somitic cells contribute to the embryonic growth of the myotome by generating fast twitch muscle fibers after somite rotation, a situation not evident in rostral somitic regions, where progenitors are required to generate hypaxial muscle during the embryonic phase of muscle growth.

We also performed cell labeling within the posterior region of somites, at both rostral and caudal somitic levels.

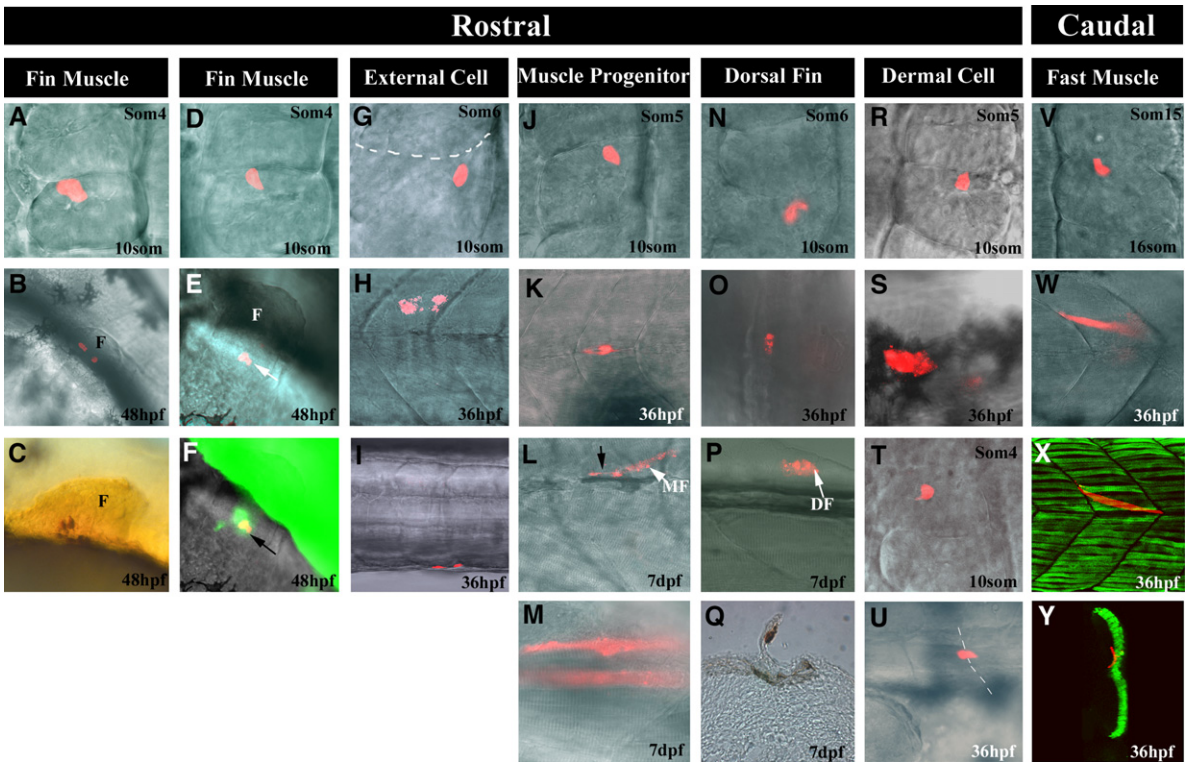

Figure 3. The Fate of Anterior Somitic Compartment Cells

(A–F) Anterior somitic cells contribute to the muscle of the pectoral fin. (A) At the 10-somite stage, two adjacent row 1 cells (see Experimental Procedures) are injected at the level of somite 4. (B and C) At 48 hpf, these cells, which contain both (B) red Rhodamine dextran and (C) Biotin dextran (revealed with streptavidin coupled with HRP), have migrated to the pectoral fin. (D–F) Similar experiments to those in as (A)–(C), carried out this time in the background of the α-actin GFP transgenic line, which expresses GFP in all skeletal muscle cells, including that of the pectoral fin. Injection of a row 1 cell (red) at the (D) 10-somite stage results in the presence of the (E) injected cell in the pectoral fin. (F) The labeled cell also expresses GFP, indicative that it is contributing to the forming pectoral fin musculature.

(G–I) Anterior somitic cells contribute to the external cell layer. (G) Row 2 cell of somite 6 immediately after labeling with Rhodamine/Biotin dextran at the 10-somite stage. The white, dashed line indicates the anterior border of the injected somite. (H) At 36 hpf, the injected cell has rotated to a lateral position, external to the (I) myotome.

(J–M) Anterior somitic cells contribute external cells that act as progenitor cells for myotomal growth. (J) An anterior somitic cell labeled at the 10-somite stage within somite 5. (K) At 36 hpf, the labeled cell has rotated to the lateral surface of the somite to form a single cell within the external cell layer. (L) At 7 dpf, the external cell has contributed to muscle fiber formation (MF, white arrow), but it is still located in its original position (black arrow). (M) High-magnification view of muscle fibers generated by the labeled external cell deeper within the myotome.

(N–Q) Anterior somitic cells contribute to the dorsal fin. (N) An anterior somitic cell labeled within somite 6 at the 10-somite stage. (O) At 36 hpf, this cell has migrated to the dorsal-most aspect of the embryo. (P) By 7 dpf, this cell is clearly evident in the forming dorsal fin (DF, white arrow). (Q) The same embryo fixed, cross-sectioned, and incubated with streptavidin coupled with HRP, which labels the Biotin dextran lineage tracer coinjected with the Rhodamine dextran fluorophore and reveals the superficial location of the labeled cell within the dorsal fin (DF).

(R–U) Row 1 and 2 cells make dermis-like cells. (R) An anterior somitic cell labeled at the 10-somite stage within somite 5. (S) At 36 hpf, the labeled cell has migrated to the dorsal-most surface of the embryo and contributed two cells in a dermal position. (T) An anterior somitic cell labeled within somite 4 at the 10-somite stage. (U) At 36 hpf, this cell has migrated to a lateral position distinct from that occupied by the external cells, outside the basal lamina of the somite spanning the vertical myosepta (dashed line) separating somites 4 and 5.

(V–Y) Anterior somitic cells also contribute to early embryonic myotome formation, by generating superficial fast twitch muscle cells. (V) An anterior somitic cell labeled at the 16-somite stage within somite 15. (W) By 36 hpf, this cell has generated a single fast twitch muscle fiber. (X) The same embryo as in (V) and (W), fixed and stained for slow twitch MyHC (green) and Biotin dextran (Red), revealing the oblique lateral positioning of fast twitch fibers generated in the caudal somite. (Y) Transverse view of the same embryo revealing the contribution to the most superficial of the fast twitch fibers, directly underneath the slow twitch muscle layer. (X and Y) Confocal renderings of a 3-somite-width view of the entire width of right-sided myotomes.

(A), (D), (G), (J), (N), (O), (R), (T), and (V), dorsal views, anterior is oriented toward the top; (I) and (S), dorsal views, anterior is oriented toward the left; (B), (C), (E), and (F), oblique lateral views, anterior is oriented toward the left; (H), (K), (L), (M), and (U), lateral views, anterior is oriented toward the left; (P), (W), and (X), lateral views, anterior is oriented toward the right; (Q) and (Y), cross-sectional views, dorsal is oriented toward the top.

These cells never contribute to the external cell layer, and, without exception, labeled cells generated fast twitch muscle fibers, deep within the medial myotome, in line with previous observations (Devoto et al., 1996) (Figure S3, n = 14). Furthermore, when anterior somite cells of rostral somites are labeled at the 18-somite stage, they generate

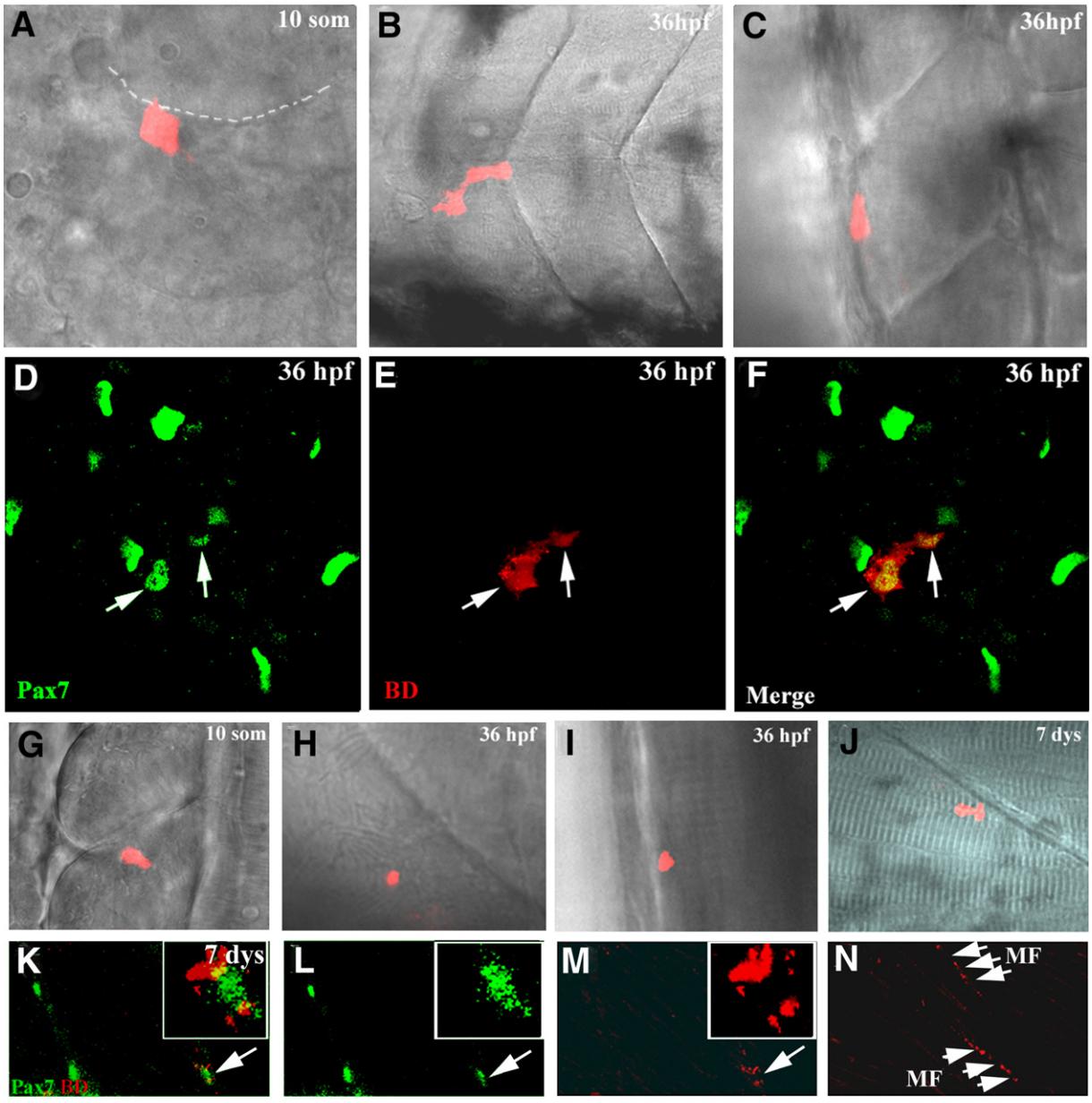

Figure 4. Anterior Somitic Compartment Cells Contribute to the Pax7-Positive External Cell Layer

(A) Iontophoretic injection of two row 1 cells at the 10-somite stage located within somite 5.

(B and C) A total of 24 hr later, at 36 hpf, these cells have migrated laterally and externally to the myotome to form large, flattened cells of the external cell layer. (A and C) Dorsal views; anterior is oriented toward the top. (B) Lateral view; anterior is oriented toward the left.

(D–F) Same embryo as in (A)–(C) stained for Pax7-positive nuclei (green) and streptavidin (red) to reveal the Rhodamine/Biotin dextran-injected cell at 36 hpf. Confocal rendering of the external cell layer and the superficial aspect of the myotome (movies of the 3D rendering are provided as Movies S3A and S3B). Coincident Biotin dextran and Pax7 localization (arrows) indicates that anterior somitic cells contribute to the external cell layer.

(G–N) Fate mapping extended to 7 days of development. (G) Anterior somitic cells labeled at the 10-somite stage; dorsal view, anterior is oriented toward the top. (H and I) (H) Lateral and (I) dorsal views of the same embryo as (G) at 36 hpf, revealing that the injected cell has rotated to the external cell layer and occupies a superficial position. (J) At 7 days, the injected cell has migrated to a deeper, fiber-adjacent position, revealed by the presence of z-bands of differentiated muscle fibers. Lateral view; anterior is oriented toward the left. (K–M) Single confocal scans of the identical larvae in (G)–(J), fixed at 7 dpf and stained for Pax7-positive nuclei (green) and streptavidin (red) to further reveal the Rhodamine/Biotin dextran-injected cell. Coincident Biotin dextran and Pax7 localization (arrow) indicates that the injected external cell undergoes self-renewal and remains Pax7 positive up to 1 week in development, despite contributing daughter muscle fibers (MF, arrows) to the myotome over this period of time. (N) MFs are evident in single confocal scans deeper in the myotome. (K)–(N) Lateral views; anterior is oriented toward the left, dorsal is oriented toward the top of single confocal scans. The insets in (K)–(M) reveal the colabeled cell at high magnification in a 3D total projection rendering of a z-stack through the entire cell.

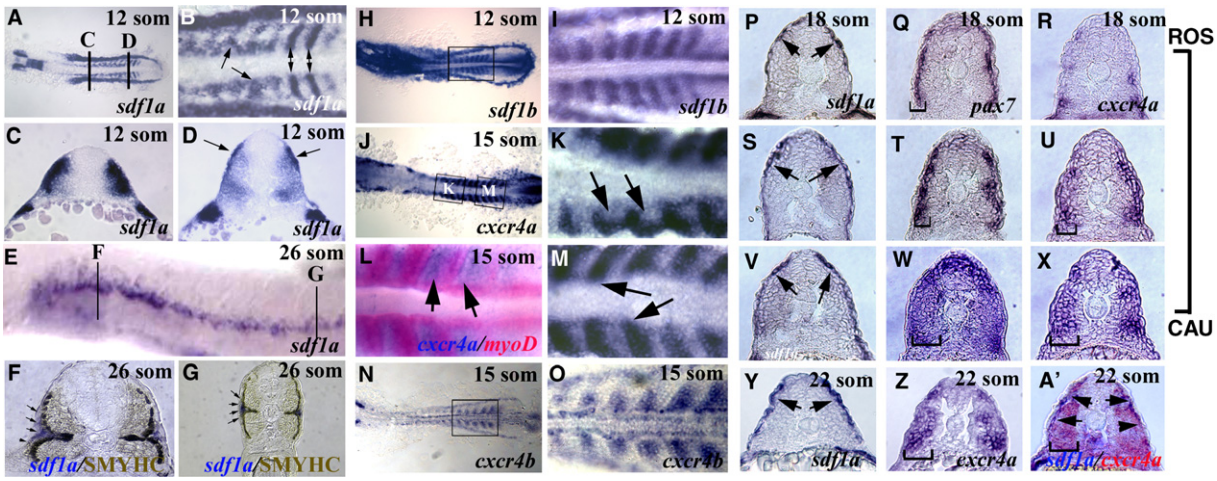

Figure 5. Expression of Components of the SDF-Signaling Pathway Prefigures Somite Rotation

(A–O) Expression of the genes encoding Sdf ligands and their receptors during somitogenesis. (A–G) *sdf1a* expression prefigures rotation and formation of the external cell layer. (A and B) *sdf1a* is expressed in the anterior domain of newly formed somites (double-headed arrow); a broader, more diffuse expression within rostral somites (arrows) is seen at the 12-somite stage. (C) Cross-section at the level shown in (A) reveals that *sdf1a* expression becomes restricted to the lateral edge of anterior somites. (D) Cross-section of newly formed posterior somites (at the level shown in [A]) reveals that *sdf1a* expression occurs throughout the somite at this level, with the expression already beginning to be restricted laterally (arrows). (E) By the 26-somite stage, *sdf1a* expression is restricted to a horizontal myoseptum-associated region. (F and G) Cross-sections at the levels shown in (E) reveal that the *sdf1a* expression at the 26-somite stage is lateral to the slow twitch muscle cells (brown) at (F) rostral and (G) caudal levels. (H) By contrast, at the 12-somite-stage, expression of *sdf1b* is localized specifically to the posterior region of the somite and the adaxial cells, the early myogenic domains of the somite. (I) High-magnification view of the region boxed in (H). (J) Expression of *cxcr4a* at the 15-somite stage reveals restriction of expression of this gene to the anterior somite. (K) High-magnification view of the region boxed in (J); lateral-restricting expression of *cxcr4a* (arrows) in rostral somites. (L) By contrast, expression of *cxcr4a* in caudal somites at this stage is restricted to the anterior somitic region (arrows), shown here in a double in situ hybridization for *myoD* expression in the posterior somite and *cxcr4a* to reveal mutually exclusive expression. (M) This anterior somitic restriction of *cxcr4a* expression (arrows) is also evident in a high-magnification view of caudal somites within the more caudal region boxed in (J). (N) Expression of *cxcr4b* is also restricted to the anterior somitic cells at the 15-somite stage. (O) High-magnification view of the region boxed in (N), showing the anterior restriction of *cxcr4b* expression that is downregulated in more rostral somites prior to somite rotation.

(P–X) Serial sections of sibling 18-somite-stage embryos hybridized with antisense probes to (P, S, and V) *sdf1a*, (Q, T, and W) *pax7*, and (R, U, and X) *cxcr4a*. Sections are arranged in a rostral to caudal order, so that sections (P)–(R) are from rostral somites, (S)–(U) are from trunk somites, and (V)–(X) are from caudal tail sections. *sdf1a* expression (arrows in [P], [S], and [V]) is restricted even in (V) tail somites to the lateral extent of the somite. By contrast, *pax7* (brackets in [Q], [T], and [W]) and *cxcr4a* (brackets in [U] and [X]), both markers of the anterior somitic compartment, remain expressed throughout the somite at caudal and trunk level somites. (Q) Expression of *pax7* begins to be restricted to lateral somitic cells only in the most rostral somites. (R) *cxcr4a* expression is downregulated in rostral somites at this stage.

(Y–A′) Sections of caudal yolk extension somites of sibling 22-somite-stage embryos incubated with antisense probes to (Y) *sdf1a* and (Z) *cxcr4a* and (A′) double in situ hybridization with both genes. Restriction of *sdf1a* to the lateral edge of the somites (arrows in [Y] and [A′]) is evident; *cxcr4a* expression remains throughout the somite (bracket in [Z] and [A′]). ROS, rostral; CAU, caudal.

fast twitch muscle fibers within 24 hr (Figure S3, n = 9). This is in line with our observation that the initially posteriorly positioned early myogenic cells come to lie in the anterior somitic region as a consequence of somite rotation (Figures 6O and 6P; Movie S2); by this mechanism, myogenic markers become expressed throughout the entire anterior/posterior extent of the somite at late somite stages (Figure 1).

SDF Signaling Is Required for Somite Rotation

We then turned our attention to the molecular basis for the process of somite rotation. A systematic search for genes expressed within the anterior somitic domain identified that both receptors for secreted cytokine Sdf, *cxcr4a* and *cxcr4b*, are expressed specifically throughout the anterior somite domain (Chong et al., 2001) (Figures 5J–5O). While *cxcr4b* expression is downregulated prior to somite rotation, *cxcr4a* exhibits an identical lateral shift in expression to that of *pax7* during the period of somitogenesis during which somite rotation is occurring, suggesting that these receptors are expressed within the lateral external cell precursors (Figures 5J–5O).

By contrast, the anterior somitic expression of the *cxcr4* ligand, *sdf1a*, becomes rapidly restricted to the lateral edge of the somite, after an initial phase of expression throughout the anterior somitic compartment of newly formed somites (Figures 5A–5D, 5P, 5S, 5V, and 5Y). The lateral restriction of *sdf1a* expression occurs immediately prior to the onset of somite rotation, at a time at which *pax7* and *cxcr4a* transcripts are present throughout the anterior somitic domain (Figures 5P–5A′). After somite rotation, *sdf1a* expression becomes confined to a central, horizontal myosepta-adjacent region of the external cell layer (Figures 5E–5G). Thus, the restriction of *sdf1a* expression to the lateral edge of the somite immediately prefigures somite rotation, and the further localization of

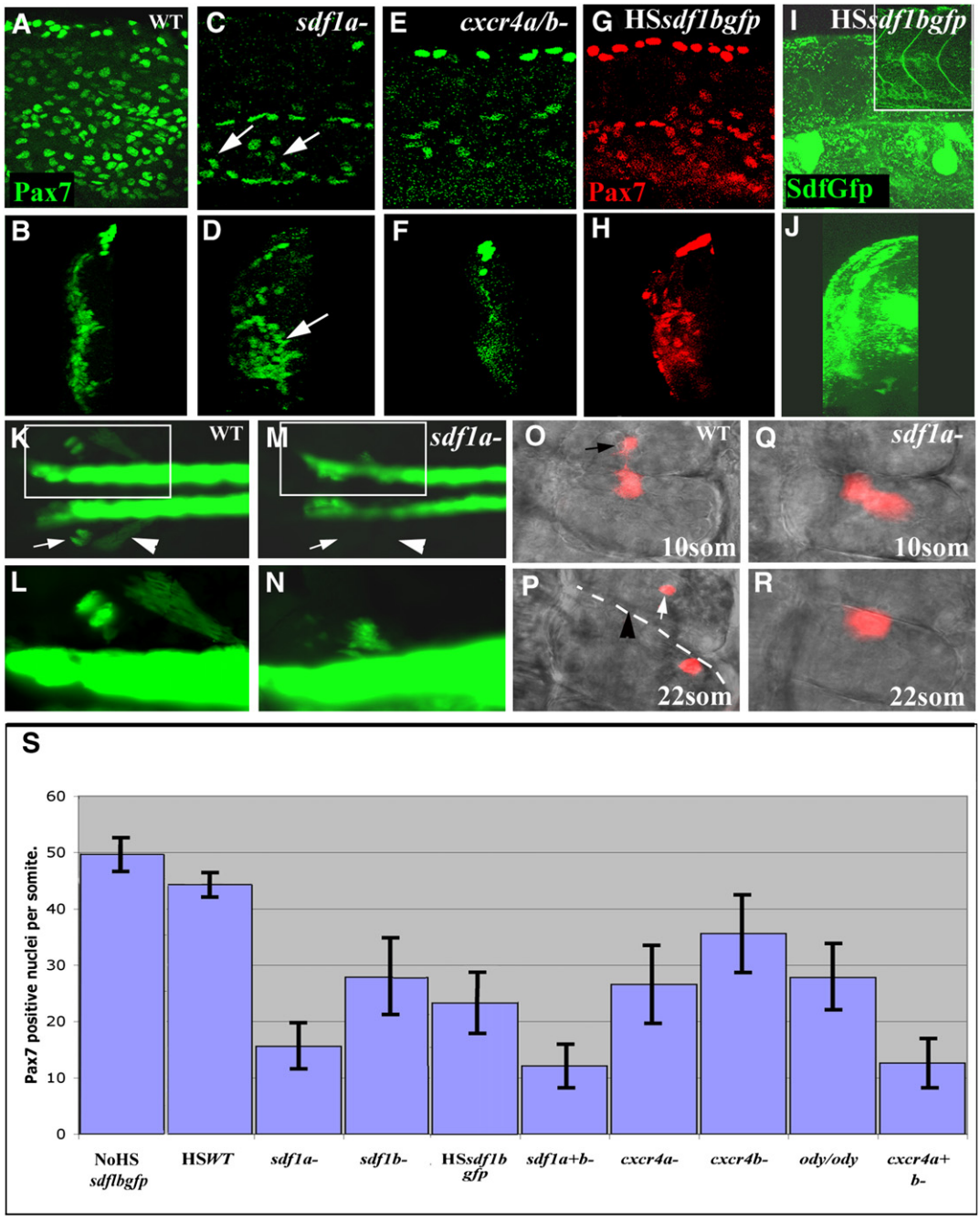

Figure 6. Sdf Signaling Is Required for Somite Rotation

(A–J) Morpholino knockdown or ectopic activation of the components of the Sdf-signaling pathway inhibits formation of the Pax7-positive external cell layer. (A and B) Confocal rendering of Pax7-positive nuclei in the external cell layer within a wild-type 26-somite embryo in a 3-somite-width lateral view (A) and in a rendering in a cross-sectional view (B). (C and D) Similar views of Pax7-positive cells within the external cell layer of *sdf1a* morphant embryos reveal that they are reduced in number and ectopically located at the anterior, myosepta-adjacent region (arrows in [C]) of the myotome. Pax7-positive cells are found throughout the mediolateral extent of the myotome (arrow in [D]). (E and F) Similar views of Pax7-positive nuclei in embryos coinjected with morpholinos against *cxcr4a* and *cxcr4b*. (G–J). Embryos transgenic for the gene encoding the Sdf1bGFP fusion protein driven by the heat-shock promoter after a 5 hr heat shock, prior to and during somite rotation. (G and H). Similar views of Pax7-positive cells (red) as in (A)–(F). (I and J) Corresponding view of the same embryo as in (G) and (H) revealing that SdfGFP expression is globally induced after heat shock. The inset in (I) represents a single confocal scan at the level of the somite within the same embryo. SdfGFP expression is clearly evident in this tissue. Images (A)–(J) are maximum projection renderings of 3-somite-width views of entire myotomes viewed in both lateral and transverse projection.

(K–N) Injection of *sdf1a* morpholinos into embryos in which GFP expression is driven off the muscle-specific α-actin promoter. Wild-type embryos ([K]; high-magnification view of boxed region, [L]) show GFP expression in the muscle of the axis as well as in pectoral fin muscle (arrow) and the posterior

sdf1a expression to the myosepta-adjacent, external cell region also precedes the concentration of Pax7-positive external cells to this identical position evident 24 hr later (Figures 5A–5G; Figure S2). By contrast, *sdf1b* expression is found within the posterior, early myogenic domain of the somite, as well as within the adaxial cells (Figures 5H and 5I). The expression of *sdf1b* is downregulated within somitic tissue prior to somite rotation (data not shown).

Sdf ligands act as chemotactic stimuli for migrating cells expressing Cxcr4 receptors in a wide variety of different cellular contexts (David et al., 2002; Doitsidou et al., 2002; Knaut et al., 2003, 2005; Li et al., 2005). Thus, the temporal and spatial regulation of the expression of the Sdf ligands and receptors during the morphogenesis of the zebrafish somite suggests a simple hypothesis. A lateralized Sdf chemotatic stimulus, provided by *sdf1a*, may induce and direct anterior somite cells, which express *cxcr4* receptors, to undergo somite rotation to form the external cell layer. In order to test this hypothesis, we made use of previously published antisense morpholino oligos designed to *sdf1a*, *sdf1b*, and *cxcr4b* (Knaut et al., 2005). We utilized the previously identified neural path-finding defects associated with the individual morphant knockdown phenotypes of each of these genes to control for the efficacy of our morpholino injections (Knaut et al., 2005) (Figure S4).

Injection of *sdf1a* morpholinos into the early zebrafish embryo resulted in a 4-fold reduction in the number of cells comprising the lateral, Pax7-positive external cell layer. The coinjection of morpholinos against *sdf1a* and *sdf1b* did not further decrease the numbers of external cells evident within these embryos. Furthermore, the Pax7-positive cells that could be detected were ectopically positioned at the anterior aspect of each somite, throughout the mediolateral extent of the myotome, suggesting a deficit in the rotational migration of these cells (n = 8, Figures 6C, 6D, and 6S). Single-cell iontophoretic cell labeling confirmed that anterior somitic compartment cells failed to migrate from their initial site of labeling in *sdf1a* morphants (n = 10, Figures 6O–6R), and these nonrotating anterior somitic cells persist, dispersed and undifferentiated, within the myotomes of *sdf1a* morphants up to 48 hpf (Figure S5). Furthermore, no increase in apoptosis could be detected in the myotomes of *sdf1a* morphants, suggesting that the reduction of Pax7-positive cells evident in these embryos was not due to death of these anterior somitic compartment cells, but rather to the loss of Pax7 expression within a large proportion of nonrotating cells (Figure S5).

In order to examine if anterior somitic compartment cells are initially correctly specified in *sdf1a* morphant embryos, we examined the expression of genes such as *pax3* (n = 29), *pax7* (n = 17), *dacD* (n = 30), and *cxcr4a* (n = 28), which specifically mark these cells prior to the onset of somite rotation. Expression of these genes is normal in prerotational stages of *sdf1a* morphants, suggesting that the anterior somitic compartment is specified correctly in these embryos. Myogenesis initiates normally in the posterior somite and adaxial domains of *sdf1a* morphants, as monitored by *myoD* expression (n = 20), and myotomal architecture appeared grossly normal when analyzed with structural markers such as myosin heavy chain (n = 13) or phalloidin (n = 22) at the end of somitogenesis. Collectively, these results suggest that Sdf signaling is required primarily for the correct location of anterior somitic compartment cells, and not for their specification and survival.

One way to directly test this model is by the use of gain-of-function experiments. If overexpressing Sdf results in the same phenotype as loss of *sdf* function, it would argue that this phenotype results from a perturbation in the movement of anterior somitic cells during rotation, rather than from a loss of a general requirement for Sdf to maintain the cellular differentiation of the cells themselves. To perform these experiments, we have made use of a transgenic line of zebrafish that expresses *sdf1b* fused to GFP under control of the heat-shock promoter (*hssdf1bgfp*), which, when heat shock is applied, results in global expression of Sdf1b, the levels of which can be directly monitored by GFP fluorescence. This line has been used to previously demonstrate that Sdf signaling controls neuronal pathfinding in the retina of zebrafish embryos (Li et al., 2005).

Heat shock of *hssdf1bgfp* embryos, prior to and during the period of somite rotation, leads to ectopically positioned Pax7-positive cells, as well as to a reduction of the number of these cells, in an similar fashion to that evident in *sdf1a* morphants (Figures 6G–6J and 6S). Furthermore, a similar heat-shock regime utilized on *sdf1a* morpholino-injected *hssdf1bgfp* embryos could not rescue the ectopic positioning and loss and Pax7 immunoreactivity evident in these morphants (n = 20). Collectively, these results suggest that it is not the requirement for Sdf signaling to direct correct differentiation of anterior somitic cells that underlies the Sdf loss-of-function phenotype, rather it is the correct localization of Sdf signaling that is critical for the morphogenesis of these cells.

Furthermore, although only a slight reduction in Pax7-positive cells was evident in embryos injected with

hypaxial muscle (arrow head). (M and N) Both of these anterior somitic compartment-derived muscles are either absent (arrow, arrowhead in left side of the embryo in [M]) or greatly reduced ([N], high-magnification view of the region boxed in [M]) in *sdf1a* morpholino-injected embryos.
(O–R), Anterior somitic compartment cells fail to rotate within the somites of *sdf1a* morphants. (O) Anterior somitic compartment and posterior myogenic compartment of the immediately rostral somite (arrow) injected at the 10-somite stage. (P) By the 22-somite stage, the anterior somitic compartment cell has rotated to the lateral edge of the somite, and the posterior cell of the more rostral somite has migrated anteriorly to the anterior somitic domain (arrow). The arrowhead marks the original position of the anterior somitic compartment cell within the somite. (Q and R) By contrast, an anterior somitic compartment cell labeled within an *sdf1a* morphant at (Q) the 10-somite stage fails to migrate laterally at (R) the 22-somite stage.
(S) Quantitation of the number of Pax7-positive cells within all morpholino injections as well as *odysseus* (ody) or *cxcr4b* mutants and transgenic heat-shock embryos. Comparisons to wild-type or noninduced transgenic controls all produced highly significant p values ($p < 0.01$, actual values are listed in Experimental Procedures). Error bars represent standard deviation.

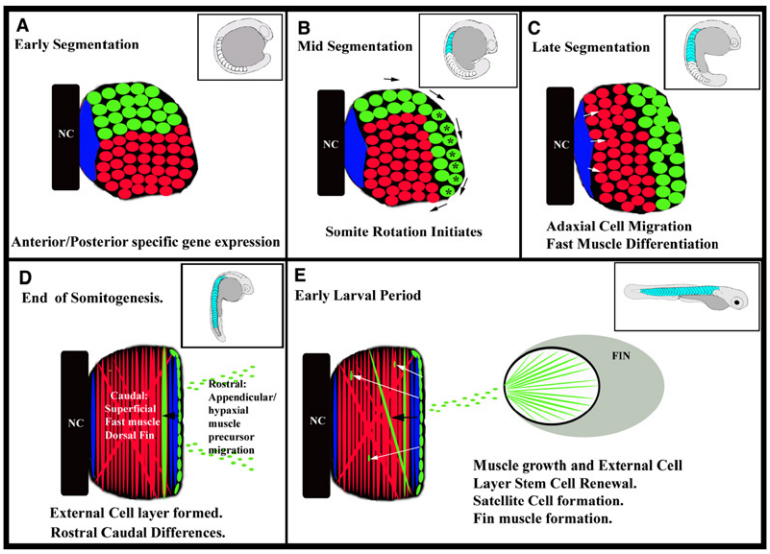

Figure 7. Somite Rotation Generates a Muscle Progenitor Cell Compartment Required for Muscle Formation during Multiple Phases of Myotomal Growth and Patterning

(A–E) Schematic representation of a dorsal view of a zebrafish somite during the ontogeny of myotome development. The stage of each schematic is illustrated in the top right-hand corner of each panel; the somites that have completed rotation are indicated in light blue. (A) Anterior/posterior gene expression is established within newly formed somites in the early segmentation-stage embryo. Adaxial cells are in blue; anterior somitic compartment cells, which express *sdf1a*, *cxcr4* receptors and *pax7*, are shown in green. Posterior "early" myogenic cells are denoted in red. NC, notochord. (B) Over the next 4–5 hr, *sdf1a* expression becomes progressively restricted to the lateral edge of the somite (denoted with an asterisk). *cxcr4*-expressing anterior somitic cells are, in turn, induced to migrate toward the lateral aspect of the somite of the myotome in response to a lateral *sdf1a* chemotatic stimulus. This occurs coincidently with an anterior migration of the posterior "early" myogenic cells. (C) All cells of the somite complete a 90° rotation, generating the Pax7-positive external cell population. After rotation, adaxial cells initiate migration from the midline (arrows). (D) Coincident with slow muscle migration, the remainder of the myotome initiates differentiation. At rostral somitic levels, hypaxial and appendicular muscle precursors, derived from the anterior somitic compartment, migrate to their targets. At more caudal somite levels, anterior compartment cells contribute directly to fast twitch muscle formation (arrow) as well as to the dorsal fin. At both levels, dermal precursors migrate external to the myotome. The remainder of the anterior somitic cells remain lateral to form the external cell layer, concentrated at the region of horizontal myosepta, where *sdf1a* expression is specifically maintained (not evident in this diagrammatic dorsal view). (E) The external cell layer is maintained throughout development and contributes muscle fibers (black arrow) to the eternal hyperplastic growth evident within fish species. The external cell layer also contributes fiber-adjacent, Pax7-positive satellite cells deep in the myotome (white arrows). Anterior somitic compartment-derived appendicular muscle differentiates within the pectoral fin.

morpholinos against either *cxcr4a* or *cxcr4b* singly or in the *cxcr4b* mutant *odysseus* (Figure 6S), coinjection of morpholinos against both *cxcr4* genes simultaneously resulted in a reduction of external cells to levels evident in *sdf1a* morphants (Figures 6E, 6F, and 6S). Loss of appendicular and hypaxial muscle derivatives are also evident in *sdf1a* (n = 21, 81%) and *cxcr4a+b* (n = 15, 93%) morphants, consistent with a failure of all cells derived from the anterior somitic compartment to undergo normal morphogenesis (Figures 6K–6N). Collectively, these results reveal that the Sdf-signaling pathway is required for the process of somite rotation in zebrafish.

DISCUSSION

The Anterior Somitic Compartment, a Lineage-Restricted Domain of the Zebrafish Somite, Is Functionally Equivalent to the Amniote Dermomyotome

Our results suggest that, while no distinct structure resembling the epithelium of the amniote dermomyotome exists in zebrafish, anterior somitic cells constitute its functional equivalent, as similar cell types such as myotomal/hypaxial muscle, muscle stem cells, and dermal progenitors originate from both structures (Figure 7). Recent studies have clearly determined that growth of the amniote myotome is dependent on a distinct population of dermyotomal progenitors that express and require Pax3 and Pax7 for their formation, and that these same somitic progenitors also give rise to satellite cells later in development (Gros et al., 2005; Relaix et al., 2005; Kassar-Duchossoy et al., 2005). Hence, clear and direct analogies can be drawn between the cell populations described in these analyses and the morphogenesis and molecular identity of the anterior somitic compartment cells of zebrafish described here. However, the utilization by zebrafish of the anterior somitic compartment-derived, Pax7-positive external cell layer to generate growth of the myotome throughout its development is clearly different from the mechanism deployed during amniote myotomal growth. Fish uniquely utilize eternal hyperplasia together with hypertrophic growth mechanisms to generate indeterminate muscle growth throughout life, whereas mammalian postnatal growth occurs exclusively via hypertrophy (Rowlerson and Veggetti, 2001). The persistence of the proliferative zebrafish external cell layer into adulthood may underlie the different modes of myotomal growth deployed in the teleost and amniote species.

The external cell layer also gives rise to Pax7-positive cells interspersed throughout the zebrafish myotome, located underneath the basal lamina of individual muscle fibers. The niche occupied by these cells is identical to that adopted by the resident stem cell population of mammalian postnatal skeletal muscle, the satellite cells, which also express and require Pax7 and are activated during injury-induced regeneration of skeletal muscle (Seale et al., 2000). Furthermore, zebrafish fiber-associated Pax7-positive cells express several other markers specific to

quiescent mammalian satellite cells such as the HGF receptor, *met* (Cornelison and Wold, 1997) (Figure S2O; data not shown), that are not expressed by the external cells layer (Figure S2; data not shown), suggesting that they form a discrete population of cells directly analogous, and possibly homologous, to mammalian satellite cells. Zebrafish show a clear capacity to regenerate in a number of different genetic and physical models of skeletal muscle injury (Rowlerson et al., 1997) (data not shown). Of clear interest for the future is to determine how the satellite cells we have identified are deployed during the regeneration of muscle tissue in zebrafish.

Our results also demonstrate a somitic origin for zebrafish dermal and dorsal fin cells. Electron microscopic studies have detailed the deposition of the collagen matrix and various steps in skin development, including the formation of the dermis in zebrafish (Le Guellec et al., 2004). These studies reveal that the zebrafish primary dermal stroma, which is composed initially of collagen secreted by the epidermis, is invaded by fibroblast-like cells, termed the "dermal endothelial cells," after 32 hpf. Based on their number and location and the timing of their development, we suggest that the cells that we have identified as originating from the anterior somitic compartment are the primary dermal endothelial cells (Le Guellec et al., 2004). Anterior somitic cells also contribute to dorsal fin formation, and the position and the frequency of which dorsal fin cells arise from the anterior somite suggests that all cells of the early embryonic dorsal fin layer may arise from this compartment. Recent observations, based solely on gene expression profiles in shark and lamprey, have suggested that the dorsal fin is a polarized structure that arises from the somites (Freitas et al., 2006). Our data provide evidence to support this assumption. Furthermore, we suggest that the loss of the dorsal fin structure that is evident during tetrapod evolution may have resulted in the co-option of anterior somitic compartment cells into the formation of the dermis of the back in amniotes, which is a more prominent cellular layer embryonically than the few scattered cells evident in zebrafish prior to the extensive dermal remodeling that is necessary for scale development in juvenile fish.

Somite Rotation and the Zebrafish Somite

The demonstration of the process of somite rotation was an unexpected finding given that there was little existing literature to suggest that such a dramatic morphogenetic rearrangement was possible within the zebrafish somite. Previous analysis has suggested that somite rotation may be a unique aspect of *Xenopus laevis* somitogenesis, and its significance has therefore been debated (Giacomello et al., 2002; Grimaldi et al., 2004; Hamilton, 1969; Keller, 2000; Wilson et al., 1989; Youn and Malacinski, 1981; Afonin et al., 2006). Although there is no evidence to suggest that rotation is a feature of amniote somite development, our results are suggestive of a wider phylogenetic distribution for the mechanism of somite rotation than previously assumed. It also raises the possibility that it may be a basal attribute of vertebrate somite formation, revealed in this instance via the use of the unique cohort of cell-labeling and imaging techniques that can be applied to the process of zebrafish somitogenesis.

In *Xenopus*, a 90° rotation of the entire somite occurs immediately after the formation of the intersomitic furrow (Hamilton, 1969; Keller, 2000; Wilson et al., 1989; Youn and Malacinski, 1981; Afonin et al., 2006), whereas somite rotation occurs mid-somitogenesis in zebrafish (Figure 7). The difference in timing between *Xenopus* and zebrafish somite rotation could be explained by the relatively precocious differentiation of muscle occurring within the *Xenopus* mesoderm, relative to zebrafish. In *Xenopus*, muscle differentiation and fiber elongation is a process that occurs coincidently with somite formation and rotation (Hamilton, 1969; Keller, 2000; Wilson et al., 1989; Youn and Malacinski, 1981). By comparison, the bulk of the zebrafish somite does not initiate muscle differentiation until mid-somitogenesis, immediately after somite rotation is complete. Thus, although it may appear that rotation of somite cells occurs in a temporally distinct phase of development in the two species, in both instances it immediately precedes the onset of differentiation of the bulk of the myotome.

Furthermore, the actual cellular process of *Xenopus* somite rotation, as described by a number of authors, is strikingly similar to the morphogenesis we describe here (Figure 7). In *Xenopus*, rotating somitic cells adopt morphologies typical of single migratory cells, with membrane protrusions and a defined polarity, characteristics of cells responding to directed, chemokine-induced migration. It has been further suggested that "leader" somite cells initiate the rotation, inducing the remainder of the cells of the somite to rotate in a coordinated manner (Keller, 2000; Wilson et al., 1989; Youn and Malacinski, 1981; Afonin et al., 2006). Previous studies have also noted that *Xenopus* somitic cells undergo somite rotation at different rates dependent on their position within the somite, a process reminiscent of the cellular behaviors we have identified during zebrafish somite rotation (Youn and Malacinski, 1981; Afonin et al., 2006). Thus, while no information is available on the expression of *sdf* and its receptors during *Xenopus* somitogenesis, the described behavior of these cells is entirely consistent with our observations on the functional requirement for Sdf signaling during somite rotation in zebrafish.

EXPERIMENTAL PROCEDURES

BODIPY-Ceramide Staining, Iontophoretic Injections, and Time-Lapse Analysis

Embryos of the AB strain were utilized for injections and stains; additional injections were performed in the α-actin GFP transgenic (Higashijima et al., 1997). *odysseus* (*ody*) mutants were a gift of H. Knaut (Harvard University). *hssdf1bgfp* transgenic fish were obtained from S. Sprague and J. Kuwada (University of Michigan). Heat shock was performed for 5 hr at 37°C, commencing at the 4-somite stage. BODIPY-Ceramide labeling was performed as described previously (Cortes et al., 2003). For iontophoretic labeling, embryos were injected by utilizing previous methods (Cortes et al., 2003) with the following

modifications. Rhodamine dextran (10,000 MW, Molecular Probes, 5 mg/ml) combined with Biotin dextran (10,000 MW, Molecular Probes, 1.5 mg/ml) were injected into cells of agarose-imbedded, segmentation-stage embryos. Anterior somitic compartment cell labelings were restricted to the two most anterior rows of cells (row 1/2 cells) within the somite, positioned at the dorsoventral level of the notochord, or above, and were imaged via the sequential capture of differential interference contrast (DIC) and fluorescent images with an ORCA digital camera (Hammumatsu). The labeled embryo was dissected free of agarose and was allowed to develop; it was then remounted in a 3% solution of methyl cellulose (Sigma) and imaged on a Zeiss Axioplan with a cooled, low-noise, high-sensitivity digital camera (Retiga Exi, Q Imaging). Individual DIC and fluorescent images were merged by utilizing IP Lab software (Scanalytics).

Gene Cloning, Morpholino Design, Injection, and Phenotypic Analysis

The *dacD* cDNA was identified from bioinformatic analysis of the zebrafish EST databases, was fully sequenced on both strands (Accession AW305923), and was compared in alignments with the three other Dachshund orthologs previously identified (Hammond et al., 2002). *sdf1a*, *sdf1b*, and *cxcr4b* morpholinos were identical in sequence to those previously described (Knaut et al., 2005). The *cxcr4a* morpholino (5′-AGACGATGTGTTCGTAATAAGCCAT-3′) was designed against the ATG region of the deposited cDNA sequence (Chong et al., 2001) (Accession AY057095). All morpholino oligos were injected at 0.25 mM and 0.5 mM as described (Cortes et al., 2003). Combined injections were performed at 0.25 mM per morpholino. Statistical analysis of Pax7-positive external cell nuclei in morpholino-treated and mutant embryos utilized a Student's t test assuming equal variance (two sample, comparison to wild-type). The p values for the different morphants are: *sdf1a*, $p = 8.15 \times 10^{-12}$; *sdf1a+b*, $p = 3.39 \times 10^{-14}$; *cxcr4a*, $p = 8.62 \times 10^{-7}$; *cxcr4b*, $p = 4.77 \times 10^{-5}$; *cxcr4a+b*, $p = 1 \times 10^{-14}$; *odysseus* homozygous mutants, $p = 3.05 \times 10^{-8}$. Additional information on methods is provided in Supplemental Data.

Supplemental Data

Supplemental Data include additional figures, movies, and Supplemental Experimental Procedures and are available at http://www.developmentalcell.com/cgi/content/full/12/2/207/DC1/.

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